



# Identification of Key Factors to Negotiate Spontaneous Spawning of *Clarias batrachus* in Confinement

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## Abstract

*Clarias batrachus* do not spawn spontaneously in confined conditions even after induction using synthetic hormones. Current hatchery techniques for seed production of the species involves fertilization of stripped eggs using ground testes suspension from a sacrificed male. The study report the elicitation of spontaneous spawning in *C. batrachus* and the successful hatching of the eggs collected there from. The mature males and females were induced using ovatide<sup>TM</sup> and released into a gently sloping drain planted with paddy saplings. The fertilized eggs were collected 24 h post injection and transferred to a flow-through device for incubation. It is observed that bilateral injection of ovatide in combination with complete exclusion of light and total simulation of the natural breeding grounds of the species have positive influence on spontaneous spawning in confinement. With finer refinements, it would be possible to have more control and success in terms of spontaneous spawning and easy collection of the eggs, which is desirable for a commercial hatchery.

**Keywords:** *Clarias batrachus*, confinement, induced, spontaneous spawning

## Introduction

The Asian catfish *C. batrachus*, popularly known as magur in India, has high aquaculture potential in Asia due to its food and medicinal value (Debnath, 2011). The species also has conservational importance as it is considered to be threatened in India

(Molur & Walker, 1998). The species do not breed in confinement like their closely allied species, *Clarias dussumieri* (Aneesh et al., 2013) and *Heteropneustes fossilis* (Nayak et al., 2001; Saha et al., 1998). Hence for culture purpose, the species is propagated by an alternative method developed around 1990. In this method, the induced ripe females are dry-stripped to release the eggs which are fertilised using the sperm suspension prepared from the testis dissected from a male (Zonneveld et al., 1988, Manickam & Joy 1989, Sahoo et al., 2008, Srivastava et al., 2012, Dhara & Saha, 2013). The males of the species need to be sacrificed since they do not express milt upon stripping. The issue of sacrificing the male for fertilising the eggs would not have been a concern from an economic point of view if at least reasonable number of healthy offsprings could be generated from a pair of brooders. Several authors have reported good fertilisation rates and hatching percentages by the above-mentioned method with the use of a variety of inducing agents (Zonneveld et al., 1988, Manickam & Joy 1989, Sahoo et al., 2008, Srivastava et al., 2012, Dhara & Saha 2013). Intriguingly, the low availability of hatchery seeds and low prevalence of commercial private hatcheries in the country contradict the claims of the success of this standardised propagation technique and farmers still largely depend on fry collected from natural waters, irrigation canal and rice fields (Pillay & Kutty, 2005). The success rates and operational skill demands of this method are not convincing enough to the farmers for adoption. Significant variation in the nature and dose of inducing hormone used as well as latency period for brooders are evident from published literature (Zonneveld et al., 1988, Manickam & Joy, 1989, Sahoo et al., 2008, Srivastava et al., 2012, Dhara & Saha 2013). It is thus apparent that high levels of experience and repeated standardisation are required for the success of this

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technique. The sustainability and repeatability of the success of this technique are questionable in the background of this scenario. Apart from the issues mentioned earlier, potential genetic improvement programs mandate preservation of the parental line which becomes impossible with a technique that requires sacrificing the male.

The major lacuna in the breeding of *C. batrachus* is the lack of a proper hatchery design conducive to induce spontaneous spawning in confinement. This bottleneck is largely due to poor understanding of the complete breeding behaviour of this species. The species is known to breed during the rainy season, in inundated paddy fields and marshy swamps. The brooders prepare pits for laying eggs and males show parental care after spawning (Pillay & Kutty, 2005). Simulation of the natural breeding environment of a species is as important for the success of captive breeding as a suitable inducing agent. This fact is clearly demonstrated by the case of eco-hatchery used for carp breeding. With this background, this study was conducted to identify the key factors necessary to induce spontaneous spawning in *C. batrachus* in confinement.

## Materials and Methods

Mature brooders of average weight 150 g were procured from a farmer in Khowai, Tripura, India in the last week of June 2015. Brooders were stocked in a cement tank of dimension 5x10 m<sup>2</sup> and fed on sinking pelleted feed (crude protein 20-22%)

procured from the Department of Aquaculture, College of Fisheries, Tripura. To confirm the taxonomic identity of the species, genomic DNA from the fin clippings of the brooders were isolated through phenol-chloroform method (Sambrook & Russell, 2001) and used for PCR amplification of mitochondrial gene Cytochrome Oxidase I (COI).

An earthen drain of dimension 29" x 19" X 11" (length x width x depth) was created in the naturally sloped terrain near the eco-hatchery of the College of Fisheries, Lembucherra, Tripura. Total 8 numbers of pits of dimension 4.8 x 3.5<sup>2</sup>" (diameter x depth) were created either at the bottom or at the junction of the bottom and lateral wall. Locally available heat hardened earthen pots of dimension 4.5 x 3<sup>2</sup>" (diameter x depth) were fixed in the pits. Plastic egg collectors of length 3.5" were provided in each earthen pot. The average slope of the drain was 6°. The drain terminated in a small circular depression (sump) of diameter 28" and depth 14" (Fig. 1). Paddy was grown for 40 days to a height of about 11" on the bottom of the drain and sump (Fig. 1a and b). The brooders were released into the sump after injection. A constant water flow of 4 l min<sup>-1</sup> was given to the drain from the highest point. To release the excess water from the drain, an outlet of internal diameter 10 mm was fixed in the sump 5" below the surface. Longer egg collectors of length 9" were given in the sump (Fig. 1c). The entire length of the drain was covered by either a net to avoid predatory birds or metal sheet to completely exclude light (see Table 1).

Table 1. Parameters of different experiment

| Experiment No. | No. of brood pair used | Water source                                                                                                                        | Drain                                                          | Injection  |
|----------------|------------------------|-------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------|------------|
| I              | 2 (2 male + 2 female)  | Water discharged from the breeding & incubation pool of eco-hatchery where <i>L. rohita</i> was simultaneously bred on the same day | Covered with net having 4 mm mesh size                         | Unilateral |
| II             | 2 (2 male + 2 female)  | Water from the pond not receiving discharged water from breeding & incubation pool of eco-hatchery                                  | Covered with metal roofing sheet; sump lined with cotton cloth | Unilateral |
| III            | 2 (2 male + 2 female)  | Water from the pond not receiving discharged water from breeding & incubation pool of eco-hatchery                                  | Covered with metal roofing sheet                               | Bilateral  |
| IV             | 2 (2 male + 2 female)  | Water from the pond not receiving discharged water from breeding & incubation pool of eco-hatchery                                  | Covered with metal roofing sheet                               | Bilateral  |

To identify the key factors, four experiments were conducted successively. Parameters such as source of water, illumination in the drain, cotton cloth lining in the sump, and method of injection were varied in each successive experiment taking cue from the earlier one. The conditions used are summed up in Table 1. For induction of spawning, both males and females were injected with ovatide™ (consists synthetic GnRH analogue and dopamine antagonist) at the rate of  $0.6 \text{ ml kg}^{-1}$  between the lateral line and dorsal side in the line of gonopore. The injections were administered at 11 AM in all four experiments. Injected brooders were released into the sump. The spawning responses were checked after 24 h of induction in each experiment. Whenever no/partial spontaneous spawning was observed during the experiment, females were stripped to collect eggs and male were sacrificed to collect the testis. The testis was ground in 0.9% NaCl solution to prepare the sperm suspension for fertilizing the stripped eggs.

A simple flow-through system was created for the incubation of eggs. Briefly, a nylon net was fixed 2" above the bottom of a perforated basket of dimension  $8 \times 4 \text{ }^2$  (diameter x height). The baskets were fitted with polystyrene foam on the collar for

floatation. Two such baskets were floated in a larger tub of dimension  $19.5 \times 9 \text{ }^2$  (diameter x height). Two holes were created 8.5" above the bottom of the larger plastic tube and the holes were jammed with plastic funnels whose mouths were covered with mosquito net to avoid the loss of eggs and hatchlings (Fig. 1f). Semi-flexible plastic tubes were fixed obliquely using bamboo sticks in each basket. Plankton free pond water stored in an overhead tank was allowed to flow at the rate of  $1 \text{ l min}^{-1}$  through these pipes, creating a slow circular current. Excess water was allowed to exit through the funnel. All the eggs were spread over the nylon net in the basket using a feather. Unfertilised eggs that turned white after some time were removed using a dropper or Pasteur pipette (Fig. 1g). The hatchlings were carefully segregated from debris with the help of a sieve. The dissolved oxygen (DO) and pH of the water used for the breeding experiments and incubation of eggs were measured regularly.

## Results and Discussion

A 650 bp length of COI gene was amplified through PCR and BLASTn homology search of the amplified product sequence showed 100% similarity with the reported sequences (accession number: GQ466399 &

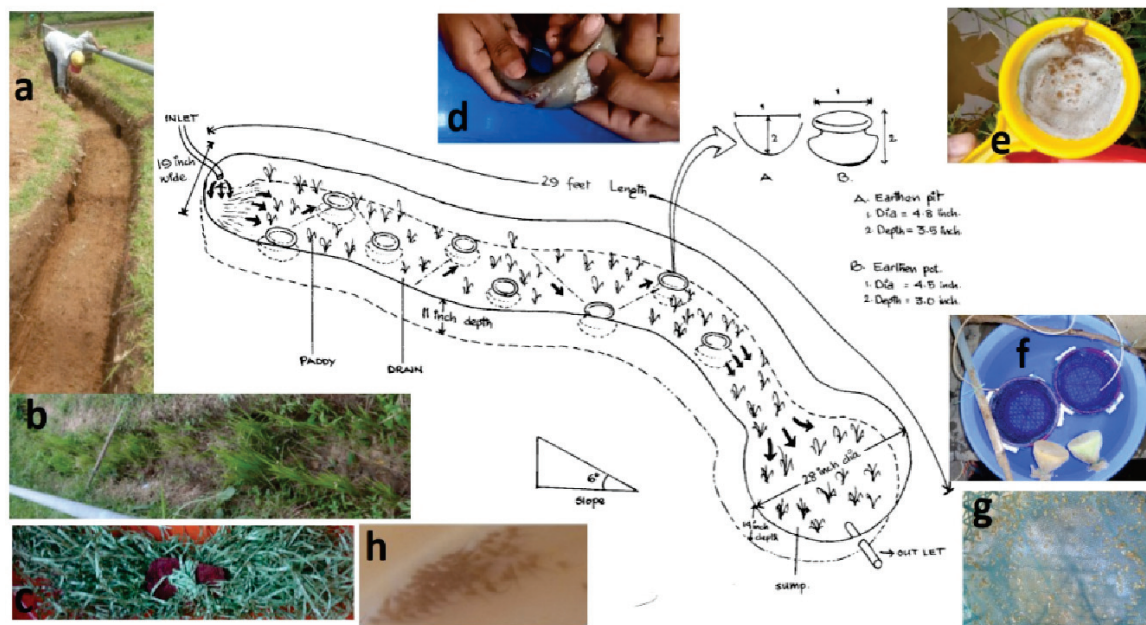


Fig. 1. Centre) Pencil sketch represents complete drain system used for experiments, a) preparation of drain, b) prepared drain with paddy plantation, c) plastic egg collectors, d) marks of mating on female body, e) collection of eggs from sump using plastic sieve, f) flow through system for egg incubation, g) greyish translucent fertilised eggs and white unfertilised eggs, h) zero-day hatchlings, A) & B) earthen pit and pot inside the drain.

GQ466403) of *C. batrachus* COI genes confirming the identity of the fish. During the entire study, DO of the water was around 7 ppm and pH was around 7.8. In experiment-I, the brooders did not spawn spontaneously till 24 h post injection. However, both the females had fully swollen and loose belly with dark red genital papillae. Eggs were stripped from the female and fertilised using sperm suspension. A total of 580 hatchlings were obtained which were further reared in the nursery. In experiment-II, the brooders did not spawn spontaneously even 30 h post injection. Moreover the artificially fertilized eggs from stripping did not hatch. There was no sign of active mating in the experiments-I & II. On the basis of results of experiment-II, the cotton lining was removed from the sump and the uprooted paddy plants were replaced with fresh saplings for experiments-III & IV. In experiment-III, spontaneous spawning was first observed 24 h post injection and the brooders were allowed to spawn for additional 6 h. Though most of the eggs were collected from the sump, eggs were also found in the entire length of the drain including the earthen pots. In the sump, most of the eggs were shed on the bottom, while few eggs were attached to the plastic egg collectors. Eggs from the sump, drain and earthen pot were collected carefully with the help of a sieve (Fig. 1e) and gently washed with water to remove mud or sand particles as much as possible. Stripping was performed since it was evident that both the female had spawned partially. Systematic enumeration of the eggs or fertilization rate was not done to avoid damage to the perivitelline membrane of the eggs (Thakur, 1976). Eggs collected from the sump and stripping were incubated in separate flow through systems. A total 623 number hatchling were recovered from eggs collected from sump and 1385 number hatchling from eggs fertilized by stripping (Fig. 1h). The females had body signs of active mating (Fig. 1d). In experiment-IV, spontaneous spawning was observed after 24 h post injection. Eggs were found in earthen pots and drain, but there were no eggs in the circular depression. Similar to experiment-III, the brooders were allowed to continue the spontaneous spawning for additional 6 h. Eggs were collected from the drain and pits. Similar to experiment-III, stripping was performed, since spawning was partial. A total of 307 and 412 numbers of hatchling were recovered from eggs collected through spontaneous spawning and stripping respectively. The four experiments were conducted serially in this study taking cue from the

results of the earlier experiment to identify the key factors to induce spontaneous spawning in *C. batrachus*. Inducing *C. batrachus* males to mate is considered difficult. Thakur (1976) observed that males initially do not show interest in mating and start indulging only after repeated nudging from the female. Signs of active mating were observed on the body of female brooders in the experiment-III and IV, apart from the presence of fertilized eggs in the breeding ground. These signs included the peeling of skin towards the middle part of the trunk in the females (Fig. 1d). Upon dissecting the males after spontaneous spawning during the third experiment, it was observed that the testis of one of the males was swollen and flaccid, releasing sperms upon gently pressing while that of the other male remained hard. This apparently indicates that a single male had participated in the mating act with both females in the experiment. None of the males in the entire study expressed milt on stripping. Nevertheless, our results show that testicular hydration of *C. batrachus* is possible in confinement if we manipulate the environment in the right orientation along with inducing agents. The nature of courtship in *C. batrachus* has been outlined in (Thakur, 1976). Polygamy is not reported in the species so far. However, in the light of parental care of eggs exhibited by the males, it would also be interesting to know whether a female engages in courtship with a different male during the same breeding season.

Complete exclusion of light is likely to be one of the key factors since it has induced spontaneous spawning in the experiments-III & IV. However, although, similar conditions of light exclusion was maintained in experiment-II, neither spontaneous spawning nor hatching from stripped eggs was observed. In experiment-II, the sump area was lined with a cotton cloth to facilitate easy collection of eggs in case of spontaneous spawning. Apparently the species has a high degree of specificity for their breeding environment and perhaps were affected by the synthetic environment. This is further evident from the fact that even eggs produced from artificial fertilization in this experiment failed to hatch. On the other hand, though no spontaneous spawning was observed during experiment-I, hatchlings could be obtained by stripping. Considering the results of all the experiments, it is clear that complete exclusion of light favours spontaneous spawning, where as deviation from natural environment, has a negative impact.

The majority of spontaneously spawned eggs in the experiment-III was collected from the sump (Fig. 1e). However, the presence of eggs in the upper stretches of the drain indicates that the fish must have migrated upstream for spawning. It is also possible that the eggs released in the drain were washed down with water flow into the pot and drain only. During experiment-III and IV, the egg collectors were used passively, and most of the eggs were recovered from the soil bottom. Thus, the adhesive nature of the eggs might be assumed to be only a mechanism to keep the eggs in a clutch for easier management by the male brooder during parental care. This indeed seems to be the case as the *C. batrachus* are not reported to actively stick their eggs to substratum unlike *Cyprinus carpio* (Jhingran & Pullin, 1985).

An accidental omission during our experiment-III led to a curious observation from our study. A miscalculation resulted in the injection of lower than required dose of ovatide during the experiment-III. This was later rectified by applying the remaining dose to the uninjected side of the brooders to avoid injection stress. With the observation of positive response during experiment-III and the failure of response in the previous, we repeated the bilateral injection in the experiment-IV and observed spontaneous spawning. It is possible that the transport of intramuscularly injected material to target organs might be faster through red muscles than white muscle. Red muscles are known to be more heavily supplied with blood vessels than white muscles (Barnabe, 2003; Val & Randall, 2005). Red muscles are mostly located along the lateral side (Altringham & Ellerby, 1999) and are generally more abundant in fishes with active and sustained movement habits (Barnabe, 2003; Val & Randall, 2005). Analysing carefully, injection of the inducing agent in carps are also usually given at just above the lateral line in the line of the first ray of dorsal fin inserting the needle 45° into the muscles probably to target the red muscle area. We speculate that bilateral injection resulted in faster distribution and better bioavailability of the hormone to the target organs in *C. batrachus* which is poorer in red muscle and induces fish for spontaneous spawning.

In a casual observation from our farm in 2010, we had observed the spontaneous spawning of *Ompok bimaculatus*. Like *C. batrachus*, *O. bimaculatus* is also difficult to spawn in confinement. The *O. bimaculatus* were kept in a pond receiving the water discharged

from the spawning and incubation pools of the eco-hatchery. This led us to suspect the involvement of pheromones in eliciting spontaneous spawning response in the *O. bimaculatus*. Sympathetic breeding response is reported in ponds in Indian Major Carp in conspecifics as a result of pheromone action (Bandyopadhyay, 1999). With this background, we had utilised the water discharged from spawning pool & hatching pool of eco-hatchery in experiment-I, in anticipation that reproductive pheromones in water from carp breeding might induce spontaneous spawning in *C. batrachus*. However, we could not observe spontaneous spawning in *C. batrachus* during experiment-I. On the other hand, in the experiment-III and IV, the brooders spawned spontaneously, even though the water was pheromone free. Sharma et al. (2014) reported a positive effect of pheromones on the hormonal profile of *C. batrachus*, though the authors did not produce any evidence for spontaneous spawning.

Systematic enumeration of eggs or determination of fertilization rate was not resorted to in order to avoid damaging the eggs, and hatchlings were counted instead. Damage to eggs during such exercises are known to occur due to dislodging of the perivitelline membrane from the adhesive eggs of magur (Thakur, 1976).

Spontaneous breeding of un-induced *C. batrachus* in pits installed in the brooder ponds was reported by Knud-Hansen et al., 1990. The brooder pond used by the author contained 100 brooders with a 1:1 male female ratio. A similar method has also been described by Pillay & Kutti, 2005. However, the methods described by these authors have a basic disadvantage of lack of control over the brooders and on the spawning act itself. The induction of spawning in these methods was under natural conditions and the nests had to be checked intermittently for eggs. Besides being tedious, this system would be practically difficult to operate in areas with heavy rainfall. Our results clearly identify the combined key factors needed to induce spontaneous spawning in *C. batrachus* in confinement. We suggest complete exclusion of light, bilateral injection of the inducing agent, constant water flow, a long drainage system with soil base and rooted vegetation for inducing natural spawning in *C. batrachus* in captive condition. More significantly our described design elicits spawning without compromising on the hatchery operator's control over the animals. The design demands no

special equipment or skills from the operator and can easily be set in the sloping terrains of farmers' backyards. It is expected that the simplicity and cost efficiency of this method would lead to better adoption of the technique by the farmers. Nevertheless, it needs to be mentioned that spawning was partial in all experiments in our study. Further refinements to facilitate easier egg collection will also be required for commercial scale applications. Further research will also be required to explain several observations on the behavioural and biological aspects of this species that we made in this study.

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